VACCINES CONTAINING ATTENUATED BACTERIA

5   The invention relates to vaccines containing attenuated bacteria.

Background to the invention

The principle behind vaccination is to induce an immune response in the host

10   thus providing protection against subsequent challenge with a pathogen. This may be
achieved by inoculation with a live attenuated strain of the pathogen (i.e. a strain
having reduced virulence such that it does not cause the disease caused by the
virulent pathogen).

15   Classically, live attenuated vaccine strains of bacteria and viruses have been
selected using one of two different methodologies. Mutants have been created either
by treatment of the organism using mutagenic chemical compounds or by repeated
passage of the organism *in vitro*. However, use of either method gives rise to
attenuated strains in which the mode of attenuation is unclear. These strains are

20   particularly difficult to characterize in terms of possible reversion to the wild type
strain as attenuation may reflect single (easily reversible) or multiple mutation
events.

Using modern genetic techniques, it is now possible to construct genetically

25   defined attenuated bacterial strains in which stable attenuating deletions can be
created. A number of site directed mutants of Salmonella have been created using
this type of technology (2, 5, 6, 12, 22, 35, 36, 37). Amongst the most
comprehensively studied attenuating lesions are those in which mutations in the
biosynthetic pathways have been created, rendering the bacteria auxotrophic (e.g. *aro*

30   genes). Mutations in these genes were described as early as 1950 (1) as responsible
for rendering Salmonella less virulent for mice. Several different auxotrophic
mutations such as *galE*, *aroA* or *purA* have also been described previously (6, 12).
Salmonella *aroA* mutants have now been well characterised and have been shown to

be excellent live vaccines against salmonellosis in several animal species. In addition, in order to reduce the chances of a reversion to virulence by a recombination event mutations have now been introduced into two independent genes such as *aroA/purA* and *aroA/aroC*. Identical mutations in host adapted strains

5 of Salmonella such as *S. typhi* (man) and *S.dublin* (cattle) has also resulted in the creation of a number of single dose vaccines which have proved successful in clinical (11, 17) and field trials (15).

In animal studies, attenuated *S. typhimurium* has been used as a vehicle for the

10 delivery of heterologous antigens to the immune system (3, 8, 32). This raises the potential of the development of multivalent vaccines for use in man (9).

Summary of the invention

15 The original aim of the work that led to the invention was the identification of novel genes that are involved in the virulence pathways of pathogenic bacteria, the identification and deletion of which may render the bacteria avirulent and suitable for use as vaccines. To identify attenuating lesions, random mutations were introduced into the chromosome of *S. typhimurium* using the transposon Tn*pho*A

20 (18). This transposon is unique in that it is engineered to identify proteins that are expressed in or at the bacterial outer membrane; such proteins may be those involved in interaction with and uptake by host tissues. By using the natural oral route of infection to screen these mutants, those with important, *in vivo* induced, attenuating lesions in genes were identified.

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One such gene identified through this work is *surA*. The *surA* gene product is known to promote the folding of extracytoplasmic proteins. Accordingly, the invention provides a vaccine comprising a pharmaceutically acceptable carrier or diluent and a bacterium attenuated by a non-reverting mutation in a gene encoding a

30 protein which promotes the folding of extracytoplasmic proteins. The vaccine has the ability to confer protection against a homologous wild type oral challenge with

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the virulent bacterium. In addition, the bacterium used in the vaccine can act as a carrier for heterologous antigens such as fragment C of tetanus toxin.

Detailed description of the invention

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Proteins that promote the folding of extracytoplasmic proteins

Periplasmic and outer membrane proteins are secreted across the cytoplasmic (inner) membrane in a mostly unfolded state, and they then fold after secretion. The
10 folding often has enzymatic assistance to catalyse the formation of bonds necessary for the protein to reach its folded state. For example, the folding often requires the participation of enzymes that catalyse the formation of disulphide bonds or enzymes that catalyse the isomerisation of prolyl bonds (peptidyl-prolyl cis-trans isomerases or PPiases).

15

One known PPiase is SurA. The inventors have now shown that mutation of the *surA* gene causes attenuation of virulent bacteria and that the attenuated bacteria are useful as vaccines.

20   SurA was first described as being essential for the survival of *E.coli* in the stationary phase (33). It is a periplasmic protein. More recently, SurA has been described as belonging to a third, new family of PPiases (30), the parvulin family. Further studies have shown SurA to be involved in the correct folding of outer membrane proteins such as OmpA, OmpF, and LamB (16, 24, 29).

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PPiases are divided into three families, the cyclophilins, FK506-binding proteins (FKBPs) and parvulins. Members of all three families have been found in *E.coli*. Apart from SurA, the parvulin family includes several proteins such as NifM, PrsA and PrtM.

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Bacteria useful in the invention

The bacteria that are used to make the vaccines of the invention are generally those that infect via the oral route. The bacteria may be those that invade and grow
5 within eukaryotic cells and/or colonise mucosal surfaces. The bacteria are generally Gram-negative.

The bacteria may be from the genera Salmonella, Escherichia, Vibrio, Haemophilus, Neisseria, Yersinia, Bordetella or Brucella. Examples of such bacteria
10 are *Salmonella typhimurium* – the cause of salmonellosis in several animal species; *Salmonella typhi* – the cause of human typhoid; *Salmonella enteritidis* – a cause of food poisoning in humans; *Salmonella choleraesuis* – a cause of salmonellosis in pigs; *Salmonella dublin* – a cause of both a systemic and diarrhoel disease in cattle, especially of new-born calves; *Escherichia coli* – a cause of diarrhoea and food
15 poisoning in humans; *Haemophilus influenzae* – a cause of meningitis; *Neisseria gonorrhoeae* – a cause of gonnorrhoeae; *Yersinia enterocolitica* – the cause of a spectrum of diseases in humans ranging from gastroenteritis to fatal septicemic disease; *Bordetella pertussis* – the cause of whooping cough; or *Brucella abortus* – a cause of abortion and infertility in cattle and a condition known as undulant fever in
20 humans.

Salmonella bacteria are particularly useful in the invention. As well as being vaccines in their own right against infection by Salmonella, attenuated Salmonella can be used as carriers of heterologous antigens from other organisms to the immune
25 system via the oral route. Salmonella are potent immunogens and are able to stimulate systemic and local cellular and antibody responses. Systems for driving expression of heterologous antigens in Salmonella *in vivo* are known; for example the *nirB* and *htrA* promoters are known to be effective drivers of antigen expression *in vivo*.
30

The invention is also particularly applicable to *E.coli*, especially

exterotoxigenic *E.coli* ("ETEC"). ETEC is a class of *E.coli* that cause diarrhoea.
They colonise the proximal small intestine. A standard ETEC strain is ATCC
H10407.

5   Infections of ETEC are the single most frequent cause of travellers diarrhoea,
causing 3-9 million cases per year amongst visitors to developing countries. In
endemic areas, ETEC infections are an important cause of dehydrating diarrhoea in
infants and young children, resulting in 800,000 deaths a year in the under fives
World-wide. In developing countries, the incidence of ETEC infections leading to
10 clinical disease decreases with age, indicating that immunity to ETEC infection can
be acquired. In contrast, naive adults from industrialized countries who visit endemic
areas are highly susceptible to ETEC infections. However, with prolonged or
repeated visits to endemic areas susceptibility to ETEC infections diminishes,
suggesting that a live attenuated approach to ETEC vaccination may prove
15 successful.

Seq. Id. No. 1 shows the sequence of the *surA* open reading frame in
Salmonella typhimurium, and Seq. Id. No. 3 shows the sequence of the *surA* open
reading frame in *E.coli*.
20

Second mutations

The bacteria used in vaccines of the invention preferably contain a mutation
in one or more genes in addition to the mutation in the gene encoding a protein which
25 promotes folding of extracytoplasmic proteins. This is so that the risk of the
bacterium reverting to the virulent state is minimised, which is clearly important for
the use of the bacterium as a human or animal vaccine. Although bacteria containing
only a mutation in a protein which promotes folding of extracytoplasmic proteins are
attenuated and the risk of reversion is small, it will generally be desirable to introduce
30 at least one further mutation so as to reduce the risk of attenuation yet further.

A number of genes that are candidates for second and further mutations are known (see e.g. ref 39). These include the *aro* genes (35), the *pur* genes, the *htrA* gene (37), the *ompR* gene (36), the *galE* gene, the *cya* gene, the *crp* gene or the *phoP* gene. The *aro* gene may be *aroA*, *aroC*, *aroD* or *aroE*. The *pur* gene may be *purA*,

5 *purB*, *purE* or *purH*. The use of *aro* mutants, especially double *aro* mutants, is preferred because such mutants have been shown to be particularly effective as vaccines. Suitable combinations of *aro* mutations are *aroAaroC*, *aroAaroD* and *aroAaroE*.

10 **The nature of the mutation**

The mutations introduced into the bacterial vaccine generally knock-out the function of the gene completely. This may be achieved either by abolishing synthesis of any polypeptide at all from the gene or by making a mutation that results in

15 synthesis on non-functional polypeptide. In order to abolish synthesis of any polypeptide, either the entire gene or its 5'-end may be deleted. A deletion or insertion within the coding sequence of a gene may be used to create a gene that synthesises only non-functional polypeptide (e.g. polypeptide that contains only the N-terminal sequence of the wild-type protein). In the case of mutations in genes

20 encoding proteins which promote the folding of extracytoplasmic proteins, the mutation generally abolishes the ability of the protein to promote such protein folding.

The mutations are non-reverting mutations. These are mutations that show

25 essentially no reversion back to the wild-type when the bacterium is used as a vaccine. Such mutations include insertions and deletions. Insertions and deletions are preferably large, typically at least 10 nucleotides in length, for example from 10 to 600 nucleotides.

30 The bacterium used in the vaccine preferably contains only defined mutations, i.e. mutations which are characterised. It is clearly undesirable to use a bacterium

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which has uncharacterised mutations in its genome as a vaccine because there would be a risk that the uncharacterised mutations may confer properties on the bacterium that cause undesirable side-effects.

5   The attenuating mutations may be constructed by methods well known to those skilled in the art (see ref 31). One means for introducing non-reverting mutations into extracytoplasmic proteins is to use transposon Tn*phoA*. This can be introduced into bacteria to generate enzymatically active protein fusions of alkaline phosphatase to extracytoplasmic proteins. The Tn*phoA* transposon carries a gene

10 encoding kanamycin resistance. Transductants are selected that are kanamycin resistant by growing colonies on an appropriate selection medium.

      Alternative methods include cloning the DNA sequence of the wild-type gene into a vector, e.g. a plasmid or cosmid, and inserting a selectable marker into the

15 cloned DNA sequence or deleting a part of the DNA sequence, resulting in its inactivation. A deletion may be introduced by, for example, cutting the DNA sequence using restriction enzymes that cut at two points in the coding sequence and ligating together the two ends in the remaining sequence. A plasmid carrying the inactivated DNA sequence can be transformed into the bacterium by known

20 techniques. It is then possible by suitable selection to identify a mutant wherein the inactivated DNA sequence has recombined into the chromosome of the bacterium and the wild-type DNA sequence has been rendered non-functional in a process known as homologous recombination.

25 Expression of heterologous antigens

      The attenuated bacterium used in the vaccine of the invention may be genetically engineered to express an antigen from another organism (a "heterologous antigen"), so that the attenuated bacterium acts as a carrier of the antigen from the

30 other organism. In this way it is possible to create a vaccine which provides protection against the other organism. A multivalent vaccine may be produced which

not only provides immunity against the virulent parent of the attenuated bacterium but also provides immunity against the other organism. Furthermore, the attenuated bacterium may be engineered to express more than one heterologous antigen, in which case the heterologous antigens may be from the same or different organisms.

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The heterologous antigen may be a complete protein or a part of a protein containing an epitope. The antigen may be from another bacterium, a virus, a yeast or a fungus. More especially, the antigenic sequence may be from tetanus, hepatitis A, B or C virus, human rhinovirus such as type 2 or type 14, herpes simplex virus,

10 poliovirus type 2 or 3, foot-and-mouth disease virus, influenza virus, coxsackie virus or *Chlamydia trachomatis*. Useful antigens include *E.coli* heat labile toxin B subunit (LT-B), *E.coli* K88 antigens, P.69 protein from *B. pertussis* and tetanus toxin fragment C.

15 The DNA encoding the heterologous antigen is expressed from a promoter that is active *in vivo*. Two good promoters are the *nirB* promoter (38, 40) and the *htrA* promoter (40).

A DNA construct comprising the promoter operably linked to DNA encoding

20 the heterologous antigen may be made and transformed into the attenuated bacterium using conventional techniques. Transformants containing the DNA construct may be selected, for example be screening for a selectable marker on the construct. Bacteria containing the construct may be grown *in vitro* before being formulated for administration to the host for vaccination purposes.

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Formulation of the vaccine

The vaccine may be formulated using known techniques for formulating attenuated bacterial vaccines. The vaccine is advantageously presented for oral

30 administration, for example in a lyophilised encapsulated form. Such capsules may be provided with an enteric coating comprising, for example, Eudragate "S" (Trade

Mark), Eudragate "L" (Trade Mark), cellulose acetate, cellulose phthalate or hydroxypropylmethyl cellulose. These capsules may be used as such, or alternatively, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is advantageously effected in a buffer at a

5 suitable pH to ensure the viability of the bacteria. In order to protect the attenuated bacteria and the vaccine from gastric acidity, a sodium bicarbonate preparation is advantageously administered before each administration of the vaccine. Alternatively, the vaccine may be prepared for parenteral administration, intranasal administration or intramuscular administration.

10

The vaccine may be used in the vaccination of a host, particularly a human host but also an animal host. An infection caused by a microorganism, especially a pathogen, may therefore be prevented by administering an effective dose of a vaccine prepared according to the invention. The dosage employed will be dependent on

15 various factors including the size and weight of the host and the type of vaccine formulated. However, a dosage comprising the oral administration of from 10^7 to 10^{11} bacteria per dose may be convenient for a 70kg adult human host.

20 **Examples**

The following Examples serve to illustrate the invention.

Brief description of the drawings

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Figure 1: Southern blot confirming the defined deletion created within *surA* in the strain BRD 1115. Lanes 1 and 2 have been restricted using the enzyme *Pst*I, lanes 3-10 have been restricted with *Sal*I. The filters have been probed using a 500 bp PCR product that contains a 500 bp fragment from the middle of the *surA* gene. Lanes 2

30 and 4 show hybridisation of this probe to a band 500 bp smaller than the corresponding wild type lanes 1 and 3. The transposon mutant BRD441 shows

hybridisation to 2 bands since the enzyme *SaI*l cuts the transposon into two. HB101 shows no hybridisation whilst the other wild type Salmonella strains show the same hybridisation as C5 when restricted with *SaI*l.

5 Figure 2: This figure shows the colonisation and persistence of BRD1115, BRD441 and the wild type C5 in the mesenteric lymph nodes (top left graph), Peyer's patches (bottom right), spleens (bottom left) and livers (top right) in BALB/c mice following oral inoculation. The x-axis is time in days and the y-axis is log_{10} CFU/ml (CFU stands for colony forming units).

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Figure 3: Three strains were constructed to evaluate the ability of mutant Salmonella strains to deliver the heterologous antigen Fragment C in the mouse. BRD1115 is the parental strain. Two plasmids encoding the Fragment C gene of tetanus toxin under the control of either the *htrA* or *nirB* promoter were introduced into the strain

15 BRD1115 to give the strains BRD1127 and 1126 respectively. Expression of fragment C was determined *in vitro* by Western blotting. These strains were then used in an *in vivo* experiment using BALB/c mice. Groups of 10 mice were immunised orally with $log_{10}8$ organisms each of the 3 strains. Serum samples were taken weekly and analysed for total antibodies against tetanus toxin fragment C. The

20 titres of anti-fragment C were determined as the highest sample dilution giving an absorbance value of 0.3 above normal mouse serum. The highest sample dilution tested was 1/6250. All mice immunised with BRD 1126 showed antibody titres higher than 6250.

25 Figure 4: Schematic showing a plasmid map of pLG339/*surA*.

Figure 5: Graph showing the survival of Balb/c mice following oral challenge with $log_{10}8$ bacteria of the three strains C5, BRD1115 and K2.

30

Example 1

This Example shows the identification of mutations in *surA* as attenuating mutations, the construction of a defined *surA* mutation and the evaluation of a *surA*

5 mutant as a vaccine (both against homologous challege and as a carrier for heterologous antigens).

Materials and methods

10 1.1 Bacteria, bacteriophage, plasmids and growth conditions

The bacteria used in this study are listed in Table 1. Bacteria were routinely cultured on L-agar or in L-broth containing 100μg/ml ampicillin or 50μg/ml kanamycin where appropriate. The bacteriophage P22HT105/1int⁻ is a high frequency transducing bacteriophage obtained from Dr Tim Foster (Trinity College,

15 Dublin). The plasmid pGEM-T (Promega Corporation, USA) is designed for direct cloning of PCR fragments and pBluescript⁰II SK+ (Stratagene Ltd, Cambridge, U.K.) is a general cloning vector. The other plasmids are described in the text.

1.2 Purification of DNA and DNA manipulation techniques

20 All DNA manipulation including Southern blotting were carried out as described by Sambrook *et al* (31). Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim (Lewes, UK) and used according to the manufacturers instructions. Chromosomal DNA preparation was prepared according to the method of Hull (13).

25

1.3 DNA sequencing

Double stranded plasmid sequencing was carried out using the Sequenase kit (Trade Mark, United States Biochemical Corporation) according to the manufacturers' instructions. Labelling of the DNA was achieved using ^{35}S-dATP

30 (Amersham, UK) and fragments separated on an 8% acrylamide/bis-acrylamide gel containing 7M urea, for 2hours at 35 mA.

1.4 DNA amplification by polymerase chain reaction

Polymerase chain reactions (PCR) were carried out with *Taq* DNA polymerase using the GeneAmp kit (Trade Mark, Perkin Elmer Cetus, USA) according to the manufacturers' instructions. Oligonucleotides were purchased from

5 the Molecular Medicine Unit, Kings College, London and the sequences are shown in Table 1. Mixtures of DNA and specific primers were subjected to multiple rounds of denaturation, annealing and extension in the presence of the enzyme *Taq* polymerase. 100 ng plasmid DNA and 1mg chromosomal DNA were added to a mixture containing 5µl 10 x buffer (100mM Tris-HCl, pH 8.3: 500mM KCl; 15mM Mg Cl$_2$;

10 0.01% gelatine(v/v)); 8µl of deoxy-nucleotide mixture (1.25mM each of deoxy-nucleotide triphosphate; dATP, dCTP, dGTP and dTTP); 1µl of a 10µM sense primer; 1µl of a 10µM anti-sense primer and 2.5 units *Taq* polymerase. This mixture is overlaid with 50µl light mineral oil (Sigma) to prevent evaporation and the tubes incubated in an Omnigene Thermal Cycler (Trade Mark, Hybaid). Amplification of

15 the DNA was performed using the following programme: 1 cycle of 95˚C for 5 minutes, 50˚C for 1.5 minutes, 74˚C for 2 minutes; 19 cycles of 95˚C for 1.5 minutes, 50˚C for 2 minutes, 74˚C for 3 minutes; 10 cycles of 95˚C 2 minutes, 50˚C for 2 minutes, 74˚C for 7 minutes.

20 1.5 Transformation of bacteria

1.5.1. Heat shock

Bacteria are rendered competent to DNA uptake by the calcium chloride method. An overnight bacterial culture was used to seed a fresh 25 ml LB broth culture (a 1:100 dilution) which was grown aerobically with shaking until the cells

25 reached mid-log growth phase (OD 650nm = 0.4 to 0.6). The cells were harvested by centrifugation at 3000 x g for 10 minutes at 4˚C. The supernatant was discarded and the pellet resuspended in 25 ml ice-cold 75mM CaCl$_2$. The process was repeated and the cells incubated on ice for 30 minutes. The cells were pelleted by centrifugation at 3000 x g for 10 minutes at 4˚C. The cell pellet was resuspended in 1.2 ml ice-cold

30 75mM CaCl$_2$ and stored on ice until needed. The cells were then competent to DNA uptake. A maximum of 20µl of the ligation mix was added to 200µl of the

competent cells and the mixture stored on ice for 30 minutes. The cells were then

subjected to heat shock by incubation in a 42°C waterbath for 2 minutes. The cells

were then transferred back to ice for a further 2 minutes. 1 ml of LB broth was added

to the mixture and the cells incubated at 37°C for at least 60 minutes to allow

5 expression of the antibiotic marker on the plasmid. 100μl aliquots of cells were

plated onto LB agar plates containing the appropriate antibiotics and incubated

overnight at 37°C.

1.5.2. Electroporation

10   Plasmid DNA was introduced into bacterial strains using electroporation.

Mid-log phase growth cultures were generated as for the heat-shock method and the

cells pelleted by centrifugation at 3000 x g for 10 minutes at 4°C. The cell pellet was

washed twice with an equal volume of ice-cold 10% glycerol and pelleted as before.

The cell pellet was resuspended in 300-500μl ice-cold 10% glycerol. Approximately

15 100 ng plasmid (or 1 μg suicide vector) in a volume not greater than 6 μl sterile water

was added to 60 μl competent cells in a pre-chilled electroporation cuvette on ice.

The plasmid was electroporated into the bacteria using a Bio-Rad Gene Pulser (Trade

Mark) with the following conditions 1.75kV, 600Ω, 25μF. 1ml LB broth was then

added to the contents of the electroporation cuvette and the mixture incubated at 37°C

20 for 90 minutes to allow the cells to recover. 100 μl aliquots of the electroporation

mix were plated out onto selection media and incubated at 37°C overnight.

1.6 P22 Transduction

         Transduction experiments were carried out using the bacteriophage P22

25 HT105/1 int⁻. Phage lysates were prepared using LB5010 as the donor strain. A 5ml

overnight culture of LB5010 was grown in L broth containing 0.2% glucose and

galactose to increase the expression of phage receptors on the cell surface. Ten fold

serial dilutions of the P22 stock were made in TMGS up to 10^{-8} (stock is

approximately 10^{10}pfu/ml). 10μl of each dilution was added to 100μl of the

30 overnight stock of cells and incubated at 37°C for 30-45 minutes to allow adsorbtion

of the phage to the cells. 3mls of top agar was added to each incubation and spread

onto L agar plates containing 100µg/ml ampicillin. The plates were incubated at 37°C for approximately 4-5 hours until plaques were visible. The dilution that gave almost confluent plaques after this length of time was the one chosen for harvesting. The plaques were harvested by scraping the top agar into 2ml of phage buffer with a

5 glass microscope slide. A few drops of chloroform were added and the phage stock stored at 4°C until needed. The recipient strain C5 was grown during the day in L broth at 37°C until late log/stationary phase. 1µl, 5µl, 10µl, 20µl, and 50µl aliquots of the new phage stock were added to 100µl aliquots of the recipient strain and incubated at 37°C for 1 hour. The cells were then spread onto L agar ampicillin

10 plates containing 5mM EGTA (to prevent phage replication) and incubated at 37°C overnight. Colonies were replated onto L agar ampicillin plates containing 5mM EGTA three times to ensure that they were free from phage. The colonies no longer had a jagged appearance thus indicating an absence of phage.

15 1.7 *In vitro* analysis of bacterial strain

1.7.1. Agglutination with antisera

Agglutination using anti-sera raised against the O antigen of *Salmonella* can be used as a rapid test not only for the integrity of the bacterial LPS but also as a diagnostic of the strain, e.g. anti-sera against the O4 and O5 antigens for

20 *S.typhimurium*. These were obtained from Murex Diagnostics Ltd (Dartford U.K.). A sweep of colonies was harvested from the growth on a plate incubated overnight, and resuspended in 100µl PBS. This sample was mixed with a drop of antisera on a glass slide and the agglutination compared with a positive and negative sample.

25 1.7.2 HEp-2 invasion assay

The HEp-2 cell line is an adherent epidermoid carcinoma derived from human larynx (ATCC CCL23). It can be cultured as a monolayer in Dulbecco's modified Eagle's medium with 10% FCS, glutamine and penicillin/streptomycin at 37°C in the presence of 5% CO_2. Confluent cells were detached from the tissue culture flasks by

30 the use of trypsin/EDTA. The cells were first washed in PBS to remove any serum that might affect the action of the trypsin. Trypsin/EDTA was then added to the

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monolayer and the cells incubated at 37°C for 5 minutes. The cells were removed from the plastic by gentle tapping on the edge of the flask. The trypsin was neutralised with 1.5 volumes of DMEM. Cells are collected by centrifugation at 1000 x g for 5 minutes. The supernatant was removed and the cell pellet resuspended 5 in DMEM. The cell pellet was counted and the concentration adjusted to give $2x10^5$cells per ml.

1 ml of the cell suspension was added to one well of a 24 well tissue culture plate (Costar 3524), three wells for each bacterial strain being investigated. The cells 10 were incubated overnight to form a confluent monolayer in the well. The cells were then washed 5 times with PBS to ensure removal of the antibiotics and 1 ml DMEM added (without any antibiotics). $1x10^7$ bacteria were added to each well and incubated at 37°C for 3 hours. The cells were washed 3 times with PBS to remove any extracellular bacteria. 1ml of DMEM containing 100µg/m gentamycin was added 15 and the cells incubated for a further 1 hour. The cells were washed 5 times with PBS. The cells were lysed by the addition of 1ml of 0.1% Triton-X-100 at 37°C for 15 minutes. The cells were further lysed by agitation with a blue pipette tip and the lysate transferred to a 1.5ml centrifuge tube. The viable bacteria that had invaded the cells were counted using the Miles-Misra drop test method (19).
20
1.8. *In vivo* analysis of bacterial strains

1.8.1. Preparation of live bacteria for immunisation of mice.

A vial of the appropriate strain was thawed from liquid nitrogen and used to inoculate a 250 ml culture of LB broth containing antibiotic where appropriate. The 25 culture was grown overnight at 37°C without shaking. The bacteria were harvested by centrifugation at 3000 x g for 10 minutes and washed once in sterile PBS. The bacteria were harvested again by centrifugation and resuspended in 5 ml sterile PBS. The concentration of bacteria was estimated by optical density at 650 nm using a standard growth curve for that strain. Based on this estimate the cell concentration 30 was adjusted with PBS to that required for immunisation. A viable count was

prepared of each inoculum to give an accurate number of colony forming units per ml (cfu/ml) administered to each animal.

1.8.2. Oral immunisation of mice with live bacteria.

5   The mice were lightly anaesthetised with a mixture of halothane and oxygen and the bacteria administered by gavage in 0.2 ml volumes using a gavage needle attached to a 1ml syringe.

1.8.3. Intravenous (i.v.) immunisation of mice with live bacteria.

10   Mice were placed in a warm chamber and 0.2 ml volumes injected into a tail vein of each mouse using a 27 gauge needle.

1.8.4. Enumeration of viable bacteria in mouse organs.

       Groups of four or five mice were sacrificed up to 7 weeks post oral

15 immunisation with three bacterial strains. Spleens, livers, mesenteric lymph nodes and Peyer's patches were removed and homogenised in 10ml sterile PBS using a stomacher (Colworth, U.K.). Dilutions of these homogenates were plated out in LB agar with kanamycin if required and incubated overnight at 37°C. The number of viable bacteria present in each homogenate was then calculated from the dilution.

20

1.9. Determination of antibody titres against fragment C.

       Serum antibody responses against fragment C were measured by enzyme-linked immunosorbant assay (ELISA) as previously described (28) using 96 well EIA/RIA plates (Costar 3590). Absorbance values were read at A_{490} and plotted

25 against dilutions (data not shown). A normal mouse serum control was added to each ELISA plate and used to define the background level response.

1.10 Tetanus toxin challenge

       Mice were challenged with 0.05 µg (50 x 50% lethal doses) of purified

30 tetanus toxin as previously described (7), and fatalities recorded for 4 days.

Results

2.1 Cloning and mapping of TnphoA insertion sites

A number of S.typhimurium TnphoA insertion mutants were previously

5   identified as being attenuated when administered orally to BALB/c mice. In addition

some of these mutants also exhibited a reduced ability to invade the cultured

epithelial cell line HEp-2. To identify the genes that had been disrupted by the

TnphoA insertion, genomic DNA was digested using Sau3A and cosmid banks

prepared from each strain. These banks were screened using TnphoA probes and

10   cosmids exhibiting homology with the 3' and 5' probes were examined. Fragments

from these cosmids were cloned into the vector pBluescript[O]II SK+. The nucleotide

sequence surrounding these insertion sites was determined and the genes identified.

Two insertions were found to be within the htrA gene (14), one in the osmZ gene (10)

and one in the surA gene.

15

The surA gene open reading frame of Salmonella typhimurium shown in Seq

Id No. 1 is 1281 bases long, encoding a protein of some 427 amino acids with a

molecular weight of 47.2Kd. This protein is virtually identical to that found in E.coli

(34), and is described as being essential for survival in long term culture (33). The

20   surA gene contains a leader peptidase cleavage site indicating that this is a

transported protein. It has now been described as belonging to a peptidyl prolyl

isomerase family, with a function to aid the correct folding of outer membrane

proteins (16, 24, 29).

25   2.2 Introduction of a defined deletion into the surA gene.

Restriction analysis and DNA sequencing of the surA gene revealed the

presence of single HpaI and SmaI restriction enzyme sites within the coding region of

the gene which could be used to generate a deletion of 400 bases. The plasmid

pGEM-T/212/213 was constructed containing a 3Kb region encompassing the entire

30   surA gene and flanking region. Digestion of the plasmid with the enzymes HpaI and

SmaI, gel purification of the large 5.5Kb fragment and re-ligation resulted in a

plasmid containing a 419bp deletion within the *surA* gene. This plasmid was designated pGEM-T/ΔsurA.

2.3 Introduction of the *surA* deletion into the chromosome of *S.typhimurium* C5.

5   The plasmid pGEM-T was digested with the two restriction enzymes *Sph*I and *Sal*I. The 2.6kb fragment containing the deleted *surA* gene was gel purified and ligated into the suicide replicon pGP704 that had previously been digested with the same enzymes. The suicide replicon pGP704 has been used previously to introduce deletions into the chromosome of *S.typhi* (4) and *S.typhimurium* (26) which lack the

10   *pir* gene, the product of which is essential for the replication of pGP704. The ligation mix was used to transform the strain SY327, an *E.coli* strain that contains the *pir* gene, and a plasmid of the expected size identified by restriction analysis. This plasmid was designated pGP704/ΔsurA. Since suicide replicons cannot replicate in *S.typhimurium* the drug resistance marker is only expressed if there has been a single

15   homologous recombination event, incorporating the plasmid into the bacterial chromosome.

The plasmid pGP704/ΔsurA was used to transform the semi-rough *S.typhimurium* strain LB5010 by the calcium chloride method. Three transformants

20   were selected on agar containing ampicillin. These single crossovers were moved from this intermediate strain into the wild type C5 using P22 transduction (20). P22 lysates were prepared from the three transductants and introduced into C5. One ampicillin resistant colony was obtained from this process. This transformant was sub-cultured twice into L-broth containing no selection and grown for 48 hours.

25   Serial dilutions of this culture were made and the 10^{-6} dilution was spread onto L-agar plates containing no selection. 500 colonies were streaked by hand on to duplicate plates, one containing agar, the other agar with ampicillin. One colony was found to be ampicillin sensitive indicating the loss of the drug resistance marker of the plasmid following a second homologous recombination event.

30

This potential *surA* mutant was confirmed as a *S.typhimurium* strain by

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agglutination with 04 and 05 antiserum. The deletion was confirmed by PCR using the primers MGR92 and MGR93, giving a 1 kb product. The deletion was also confirmed cloning the PCR product into the vector pGEM-T to give the plasmid pGEM-T/92/93, and sequencing across the deletion using the primers MGR130 and

5   135. Figure 1 shows the results of probing *PstI* and *SalI* digested genomic DNA from C5 and the *surA* mutant strain with a PCR product obtained from the wild type C5. The band seen in the *surA* mutant track is approximately 400 bases smaller than that seen in the wild type. This deleted strain was designated BRD1115.

10 2.4 Characterisation of the strain BRD1115

2.4.1 *In vitro* analysis of the invasion of cultured epithelial cells

The strain BRD1115 was tested for its ability to invade the cultured epithelial cell line HEp-2. The levels of invasion were found to be reduced by 80% in comparison to the wild type strain C5. The transposon mutant BRD441 showed a

15 90% reduction in invasion compared to C5.

2.4.2. Evaluation of the *in vivo* properties of BRD1115 in BALB/c mice.

2.4.2.1. Determination of oral and i.v. LD50's

The oral and i.v. LD $_{50}$'s of BRD 1115, C5 and BRD441 were calculated

20 using the mouse susceptible strain BALB/c. 5 mice per group were inoculated either orally or i.v. with doses ranging from $\log_{10} 4$ to $\log_{10} 10$ orally and $\log_{10} 1$ to $\log_{10} 5$ i.v. Deaths were recorded over 28 days and the LD $_{50}$'s calculated by the method of Reed and Meunch (27). BRD1115 was determined to show nearly 5 logs of attenuation orally and 3.5 logs i.v compared to C5. BRD441 showed 4.5 logs attenuation orally

25 and 1 log i.v.. The results are presented in Table 2.

2.4.2.2. Persistence of strains in the organs of BALB/c mice following oral inoculation

Groups of 4 BALB/c mice were orally inoculated with $\log_{10} 8$ organisms of the

30 three strains. Mice were killed at days 0,1,4,7,10,16,21 and 28 and the organs examined for bacterial load. The wild type strain C5 colonised the spleen, liver,

mesenteric lymph nodes and Peyer's patches in high numbers ($>\log_{10}4$ cfu/ml), eventually resulting in the death of the animals. BRD 1115 and BRD 441 on the other hand persisted in the liver and spleens for more than 40 days in low numbers ($<\log_{10}2$ cfu/ml). These results are presented in Figure 2.

2.5. Evaluation of BRD1115 as a potential vaccine strain

2.5.1. BRD1115 protects against homologous challenge

Groups of BALB/c mice were orally immunised with $\log_{10}8$ organisms of BRD1115 and challenged with the wild type strain C5 at 4 weeks and 10 weeks post inoculation. The mice were challenged with $\log_{10}4$ to $\log_{10}10$ organisms C5 and a new oral LD_{50} calculated. The levels of protection are presented in Table 3, showing $\log_{10}4$ protection after 4 weeks and $\log_{10}5$ after 10 weeks.

2.5.2. BRD1115 as a potential carrier strain for heterologous antigens

Two plasmids encoding the C fragment of tetanus toxin were introduced into two isolates of BRD1115 by electroporation. The plasmids are pTET*nir*15 (38) in which fragment C is under the control of the *nirB* promoter, and pTET*htrA* in which fragment C is under the control of the *htrA* promoter. The plasmids were found to be maintained at levels greater than 90% in BRD1115 even when the selection pressure of ampicillin was removed from the growth medium. *In vitro* expression of fragment C was determined by Western blotting. The strains were cultured under both inducing (42°C for BRD 1126 and anaerobiosis for BRD 1127) and non-inducing conditions (37°C for BRD 1126 and aerobiosis for BRD 1127). A higher level of expression was seen for both strains under inducing conditions with BRD 1127 showing higher levels of fragment C expression than BRD 1126.

Groups of 10 BALB/c mice were orally immunised with $\log_{10}8$ organisms and bled weekly. The titres of anti-fragment C antibodies present in the serum of each animal was determined by ELISA. The titres were determined as the reciprocal of the highest sample dilution giving an absorbance of 0.3 above normal mouse serum. The results are presented in Figure 3.

Four weeks post immunisation the mice were challenged with $50LD_{50}$'s of tetanus toxin subcutaneously and the deaths noted over 4 days. The results are presented in Table 4, showing that 100% protection was given after immunisation with BRD1127 (fragment C under the control of the *htrA* promoter) and 60% protection after immunisation with BRD1126 (under *nirB* promoter). No naive mice survived the challenge.

<u>**Example 2**</u>

This Example confirms that the mutation in *surA* is responsible for the attenuation. This was determined by complementation of the deleted gene with an intact version of the gene expressed on a plasmid. The complemented strain was as virulent as the wild-type organism given orally to mice.

Materials and Methods

3.1 Construction of plasmid containing the intact *surA* gene

pLG339 (41) is a low copy number plasmid based on pSC105. A 3kb fragment of the plasmid pGEM-T/212/213 (section 2.2) containing the intact *surA* gene and flanking region was cloned into the *SphI/SalI* sites of the plasmid pLG339 to create the plasmid pLG339/*surA*. A schematic of this plasmid is shown in Figure 4.

3.2 Introduction of the plasmid pLG339/*surA* into defined mutant strain BRD1115

The plasmid was electroporated into electrocompetent BRD 1115 as previously described in section 1.5.2. Transformants containing the plasmid were selected by plating the electroporation mix onto agar plates containing 15µg/ml kanamycin. Plasmid DNA was recovered from a single colony of this transformation and checked for identity by restriction analysis. This strain was called K2.

3.3 Plasmid stability within the strain K2.

The ability of the intact *surA* gene on the plasmid to complement the action of the deleted *surA* gene in the chromosome relies on the plasmid being retained within the bacterial strain. The plasmid contains the gene encoding resistance to the

5 antibiotic kanamycin. Culturing the strain in the presence of the antibiotic should ensure that the plasmid is retained. However it is important that the plasmid be retained in the absence of the antibiotic selection as antibiotic selection is not possible *in vivo*.

10 A single colony of the strain K2 was inoculated into duplicate 10 ml cultures of L broth with and without kanamycin. The cultures were grown with shaking at 37°C for a total of 72 hours. Samples were taken at 30 and 48 hours post inoculation and serial dilutions plated onto L agar plates with and without kanamycin. The cultures were diluted 1/100 into Fresh L broth with and without

15 kanamycin and cultured for a further 24 hours. Dilutions of the culture were again plated out onto L agar plates with and without kanamycin. Numbers of colony forming units (cfu) were recorded and are reported in Table 5.

3.4 Oral immunisation of mice with the strain K2.

20 The strain K2 was grown as described in 1.8 and used to challenge orally groups of 5 Balb/c mice (as previously described) with a dose range from 10^4 to 10^{10} /dose. Deaths were recorded over 28 days and the LD_{50}s calculated according to the method of Reed and Meunch (described in 2.3.2).

25 **Results**

4.1 Strain

The plasmid pLG339/*surA* was recovered from the strain K2 and digested with the two enzymes *Sph*I and *Sal*I. Separation of the resultant bands by agarose gel

30 electrophoresis revealed the correct sized bands of 6.2 and 3 kb.

4.2 Plasmid Stability

The presence of the plasmid pLG339/*surA* was investigated in the strain K2. The results show that in the absence of antibiotics the plasmid is retained by the bacteria. In these studies, at least 82% of the bacteria retain the plasmid when grown
5 without antibiotics. This suggests that this plasmid should be maintained when the bacteria are used to infect mice.

4.3 Complementation data

Groups of 5 Balb/c mice were orally challenged with various doses of the
10 putative complemented strain K2. The oral LD_{50} of the complemented strain K2 was calculated to be $log_{10}4.35$ compared to that of $log_{10}4.17$ for the parental strain C5.

Deaths of the mice within the group of mice challenged with $log_{10}8$ bacteria of the three strains C5, BRD1115 and K2 are represented in Figure 5. Although the
15 *surA* gene expressed from the plasmid appears to commplement the defined mutation *in vivo*, the apparent delay in the time to death (when compared to the wild type parent strain) suggests the level of *surA* expression may be reduced in the strain K2.

Tables

Table 1: Bacterial strains, plasmids and oligonucleotide primers used in this study

5 Bacterial strains	Properties	Source or ref
E.coli		
SY327	λ*pir* lysogen	Miller V.L.(23)
S.typhimurium		
10 LB5010	semi-rough	the inventor laboratory
C5	wild type	C.Hormaeche, Cambridge, U.K.
BRD441	TnphoA mutant, kan^R	Miller I (21)
BRD 1115		this study
BRD 1126	amp^R	Oxer M.D. (25)
15 BRD 1127	amp^R	in press
Plasmids		
pBluescript^{O}II SK+	amp^R	Stratagene Ltd
pGEM-T	amp^R	Promega Corp.
20 pGP704	amp^R	Miller V.L. (23)
pGEM-T/212/213	amp^R	this study
pGEM-T/ΔsurA	amp^R	this study
pGP704/ΔsurA	amp^R	this study
pGEM-T/92/93	amp^R	this study
25 pTET*nir*15	amp^R	Oxer M.D. (25)
pTET*htr*A	amp^R	in press
Oligo Primers		
MGR 92	TCGGCACGCAAGAAATGT	Kings College, London
30 MGR 93	AGACGACCAGTTCAATCG	" " "
MGR 130	CGATGGGCTGAACTATTC	" " "
MGR 135	TATGCAGCTTCGTTAGCG	" " "

Table 2: The oral and i.v. LD $_{50}$'s of the three strains C5, BRD 441 and BRD 1115

35 were determined in BALB/c mice. Groups of 5 mice were immunised with doses

ranging from $\log_{10}4$ to $\log_{10}10$ cfu of the strains BRD 441 and BRD 1115, and doses

$\log_{10}1$ to $\log_{10}5$ of the strain C5. The results are presented in the following table.

	Strain	oral LD$_{50}$ (log$_{10}$ cfu)	i.v.LD$_{50}$ (log$_{10}$ cfu)
40	C5	4.16	<1.87
	BRD 441	8.62	2.46
	BRD1115	8.98	5.22 .

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Table 3: The ability of the defined *surA* mutant strain to confer protection against homologous challenge with the wild type strain C5 was determined. Groups of 5 BALB/c mice were orally immunised with $\log_{10}8$ organisms of the strain BRD1115 then challenged with $\log_{10}4$ to $\log_{10}10$ of the mouse virulent strain C5 either 4 or 10 weeks post inoculation. The new LD_{50} was then calculated and the results presented in the table below.

Immunising strain	oral LD_{50} of C5		protection (no of LD_{50}'s)
	4 weeks post immunisation	10 weeks post immunisation	
BRD1115	8.58		~3800
none	4.74		
BRD 1115		9.51	~4800
none		4.68	

Table 4: Three groups of 10 mice were immunised with the strains BRD1115, BRD1126 and BRD1127 and then challenged 4 weeks post immunisation with 50 LD_{50} doses of tetanus toxin subcutaneously. Deaths were noted over 4 days. The numbers of mice surviving the challenge are presented in the table below.

Strain	Survivors after challenge
BRD 1115	0/10
BRD 1126 (*nirB*)	6/10
BRD 1127 (*htrA*)	10/10

Table 5: The numbers of bacteria (cfu) present in the cultures of the complemented strain K2 following culture in L broth with and without the antibiotic kanamycin were calculated. The cultures were then plated onto L agar with and without kanamycin to show presence of the plasmid pLG339/*surA*. The results are presented as a total number and also the kanamycin resistant colonies as a percentage of the total bacteria present.

Kanamycin in broth	Kanamycin in agar	numbers of bacteria (cfu/ml)					
		30 hours	(%)	48 hours	(%)	72 hours	(%)
++	++	4.75×10^7	(95%)	6×10^7	(71%)	8.25×10^7	(82.5%)
++	- -	5×10^7		8.5×10^7		10×10^7	
- -	++	5.25×10^7	(124%)	9.5×10^7	(111%)	6×10^7	(89%)
- -	- -	4.25×10^7		8.5×10^7		6.75×10^7	

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